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Differential Use of the Two High-Oxygen-Affinity Terminal Oxidases of *Brucella suis* for In Vitro and Intramacrophagic Multiplication

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Expression of the high-oxygen-affinity cytochrome *cbb3* and cytochrome *bd* ubiquinol oxidases of *Brucella suis* was studied in vitro and in the intramacrophagic niche, which was previously proposed to be oxygen limited. The cytochrome *cbb3* oxidase was exclusively expressed in vitro, whereas the cytochrome *bd* oxidase was preferentially used inside macrophages and contributed to intracellular bacterial replication.

Brucellosis, an anthropozoonosis encountered worldwide, is caused by the gram-negative intracellular pathogens *Brucella* spp., which utilize macrophages to multiply inside a specific niche (12) and to spread throughout the organism. Low levels of nutriments and oxygen, as revealed by the analysis of the intramacrophagic virulome (11), are major features of the *Brucella suis* replicative niche. Phagosomes of stimulated macrophages are known to have oxygen concentrations which are lower than those found in the extracellular environment (9). Furthermore, granulomatous structures generated by the immune system during localized infection within livers, spleens, or brains of patients are characterized by oxygen deficiency (2, 20, 21).

Pathogenicity of brucellae and chronicity are due to the ability of the pathogen to adapt to the environmental conditions encountered in its replicative niche. To perform this task, *Brucella* has to modify its gene expression profile to rapidly adapt to the intracellular conditions. To this end, the bacteria induce a set of virulence genes, the main one being *virB*, encoding a type IV secretion system (17). Expression of genes involved in adaptation to oxygen-limited conditions appeared to be crucial for intramacrophagic survival of *Brucella*. A previous study identified a *cydB* mutant of *Brucella abortus* lacking the cytochrome *bd* oxidase with high affinity for oxygen as being highly attenuated in the mouse model of infection (6). Complete genome sequences (5, 8, 18) have revealed that *Brucella* possessed the locus *ccoNOQP*, potentially encoding another high-oxygen-affinity oxidase, the cytochrome *cbb3*-type terminal oxidase. We also identified a putative transcription regulator of the FixK/Fnr family in *B. suis*. The present study was undertaken to investigate (i) expression of the two operons encoding the cytochrome *bd* and cytochrome *cbb3*-type terminal oxidases in vitro as well as in bacteria obtained from infected cells and (ii) their respective roles in intracellular multiplication.

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Expression of FnrN, a potential oxygen sensor in *B. suis*, is increased in bacteria grown under microaerobiosis. Analysis of *B. suis* and *Brucella melitensis* genome sequences detected a unique copy of a gene predicted to encode a transcriptional regulator that possessed distinctive features of oxygen sensors. In a phylogenetic study (14), the *B. suis* Fnr-like factor was found to cluster with the members of the FnrN class, which comprises regulators of some rhizobial species. They have in common the conserved cysteine motif of Fnr, which is the general transcription factor of *Escherichia coli* under anaerobic conditions. These amino acids are ligands to the iron-sulfur cluster, whose oxidative state drives the transcriptional activity of Fnr (7). The 5′ region and the upstream sequence of *fnrN* were produced by PCR from *B. suis* 1330 (ATCC 23444) genomic DNA with primers FnrN5′ (TGGTACCCGGCTGA TTTCGC) and FnrN3′ (GGATTTACGGGAGCTACGGC). Expression of *fnrN* in *B. suis* was monitored using a transcriptional fusion of the promoter region located on the 305-bp KpnI-HindIII fragment from the PCR product, with the promoterless *gfp* gene of the pBBR1-CGFP plasmid construct. This plasmid was obtained after replacement of the kanamycin resistance gene by a chloramphenicol resistance cassette (from plasmid pBlueCm-2) in plasmid pBBR1-KGFP (13). Fluorescence intensity in cultures grown in tryptic soy broth until mid-log phase (approximately 4×10^9 bacteria ml^{-1}) was quantified with a FACScalibur scanner (Becton Dickinson, San Jose, CA) (10). To perform cultures under microaerobiosis, 3-ml cultures of brucellae were grown in loosely capped 10-ml tubes placed in a jar with GENbox generators (bioMérieux, Marcy l'Etoile, France) of microaerobic atmosphere (oxygen concentration ranging from 6.2 to 13.2% after 1 h). The jar was shaken at 80 rpm to keep the bacteria in suspension. The *fnrN* promoter was active in *B. suis* cultures under aerobic conditions, exhibiting an 8- ± 1-fold-higher fluorescence than the promoterless control plasmid pBBR1-CGFP, and this activity was increased to a 30- ± 5-fold-higher fluorescence under microaerobic conditions (not shown). We then analyzed the expression of *fnrN* in bacteria multiplying inside human THP-1 macrophage-like cells. Briefly (10), 4×10^6 VD3-differentiated cells were infected with 1 ml of bacteria suspension, corresponding to a multiplicity of infection of 80. At 48 h postin-

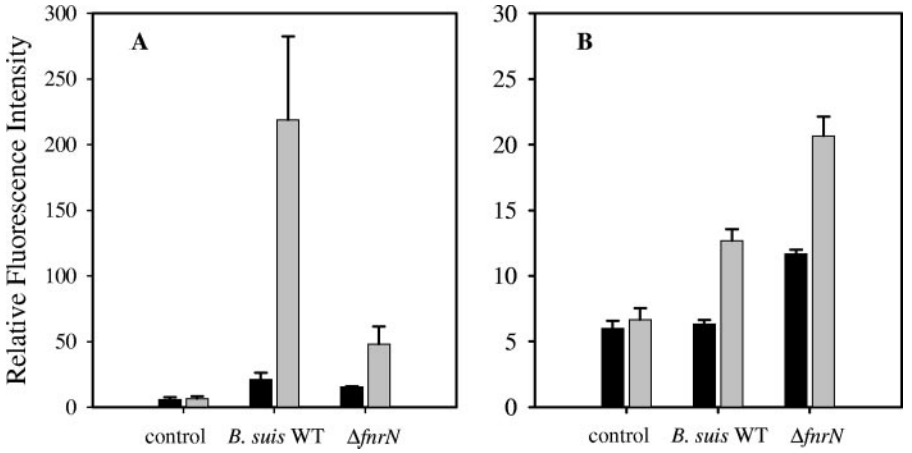

FIG. 1. Expression of the *cbb3*-type cytochrome oxidase and the cytochrome *bd* ubiquinol oxidase in wild-type (WT) and Δ*fnrN* mutant strains of *B. suis*. In vitro expression levels of the *ccoNOQP* (A) and *cydDCAB* (B) promoters were determined by flow cytometry analysis of the *gfp* construct under aerobic (black bars) and microaerobic (gray bars) conditions. The control is wild-type *B. suis* transformed with native plasmid pBBR1-CGFP. Standard errors are reported for the means from three independent experiments.

fection (p.i.), the cells were scraped off, washed with phosphate-buffered saline (PBS), and lysed in 1 ml 0.1% Triton X-100 by incubation for 10 min on ice. After centrifugation (1,000 rpm, 5 min) to pellet cellular debris and nuclei, bacteria contained in the supernatant were recovered by centrifugation (13,000 rpm, 10 min) and diluted in 500 μl PBS for flow cytometry analysis. Measurement of fluorescence intensity revealed that the intracellular promoter activity was 10- ± 2-fold higher than that of the negative control, which was not statistically different ($P = 0.5$; Student's *t* test) from that obtained in vitro under aerobic conditions. This result can be interpreted according to two hypotheses: first, *fnrN* may not be involved in the transcriptional regulation within intracellular brucellae, and second, oxygen tension may not differ significantly inside and outside the host cell. To discriminate between these two hypotheses, we decided to study expression of the two high-oxygen-affinity terminal oxidases in the wild-type and *fnrN* mutant strains of *B. suis*. The Δ*fnrN* mutant was obtained by allelic exchange between chromosomal *fnrN* and a PCR product (see above) of this gene cloned into a suicide plasmid, with a deletion of a 480-bp HindIII fragment which was replaced by the kanamycin resistance gene from plasmid pUC4K (10). Levels of intracellular expression of high-oxygen-affinity terminal oxidases would be indicative of the intramacrophagic oxygen tension.

FnrN specifically activates in vitro expression of the cytochrome *cbb3*-type terminal oxidase. Expression of the cytochrome *cbb3*-type and cytochrome *bd* oxidases was analyzed by measurement of green fluorescent protein (GFP)-mediated fluorescence under control of the *ccoNOQP* and *cydDCAB* promoters, respectively. The transcriptional *gfp* fusions to the *cco* and *cyd* promoter regions comprised 290 bp and 1 kb of upstream sequences of *ccoN* and *cydD*, obtained by PCR with primers 5PRCcoN (GACCGCTCGAGACGGCTACAGGATCAGCAAG) and 3PRCcoN (ACGCACGGTACCGTTGCGATGACGCCATAAC) and with primers 5PRCyd (CCGCTCGAGACAGCAAGGAGTTGCCTTC) and 3PRCyd (GGTACCGCTGCATAAGCCAGAAGGGC), respectively. XhoI and KpnI restriction sites in the 5′ and 3′ primers allowed direct cloning into the pBBR1-CGFP plasmid.

Transcription of the *ccoNOQP* operon was strongly increased under microaerobic conditions (10-fold; $P = 0.037$). As induction by microaerobiosis was significantly reduced in the Δ*fnrN* mutant (Fig. 1A) and was not different from results obtained under aerobic conditions ($P = 0.075$), microaerobic activation depended strictly on FnrN. The *ccoNOQP* operon may therefore represent one of the potential targets of *B. suis* FnrN, as underlined by the finding of the perfect "FNR box" TTGAT N_4 ATCAA located at a proper distance from ATG of *ccoN* (3), the transcription initiation site being unknown.

In the wild-type strain, cytochrome *bd* oxidase expression was not detected under aerobic conditions, as the fluorescence intensity measured was the same as that obtained with the control (Fig. 1B). Slight induction was observed under microaerobiosis (twofold; $P = 0.01$). With both oxygen concentrations tested, the disruption of *fnrN* increased ($P < 0.01$) *cydDCAB* transcription levels by a rather similar factor compared to those observed in the wild type (1.8-fold under aerobic conditions and 1.6-fold under microaerobic conditions) (Fig. 1B). These results indicated that in contrast to the cytochrome *cbb3*-type oxidase, the cytochrome *bd* oxidase was poorly expressed in vitro under microaerobic conditions and that the *fnrN* regulator repressed its expression.

The cytochrome *bd* oxidase is the sole high-oxygen-affinity terminal oxidase expressed inside macrophages. Activity of the promoters in bacteria multiplying within human THP-1 macrophage-like cells was analyzed as described above for the *fnrN* promoter. In the macrophage, the *ccoNOQP* promoter was inactive (Fig. 2), as no fluorescence peak could be observed, and the *fnrN* deletion had no effect (not shown). In contrast, the *cydDCAB* promoter was found to be expressed similarly in the presence (Fig. 2) or absence (not shown) of the native *fnrN* gene. These results suggested that inside the cells, *B. suis* specifically utilized the cytochrome *bd* oxidase, via another, unknown mechanism of regulation.

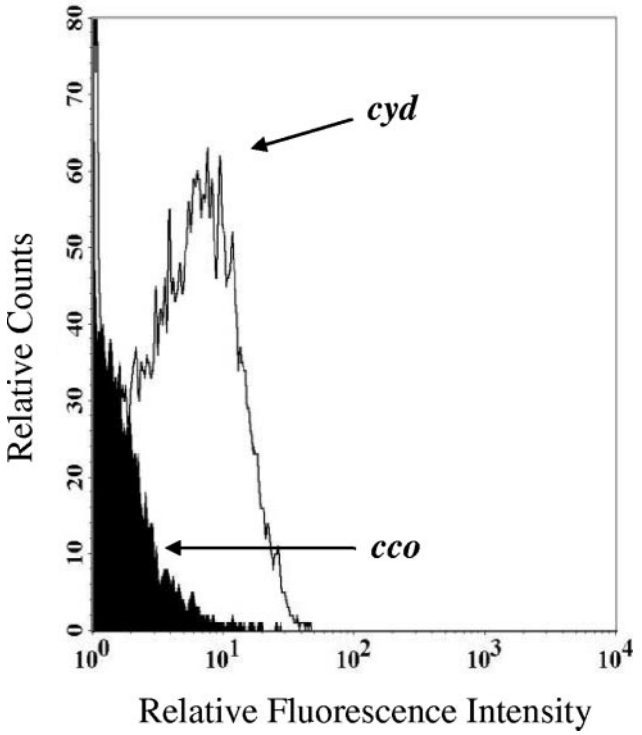

FIG. 2. Expression of intramacrophagic terminal cytochrome oxidases. Flow cytometry analysis of expression of the *cyd* and *cco* promoter-*gfp* fusions in wild-type *B. suis* obtained from infected macrophages is shown. The control curve corresponding to intracellular bacteria containing native plasmid pBBR1-CGFP was identical to that of p*cco*-CGFP and is omitted for clarity.

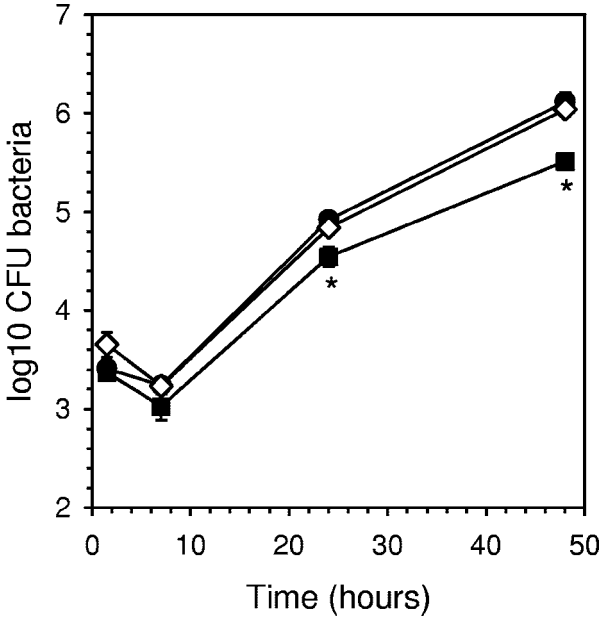

FIG. 3. Intracellular growth of *B. suis* mutants in human THP-1 cells. Cells were infected with the wild-type strain (●) or the *ccoN* (◇) or *cydB* (■) mutant. Experiments were performed in triplicate, and error bars represent the standard errors of the means. Asterisks indicate statistical significance ($P = 0.03$ and $P = 0.01$ at 24 h and 48 h p.i., respectively, as determined by Student's *t* test).

The cytochrome *bd* oxidase but not the cytochrome *cbb3*-type oxidase participates in intracellular growth of *B. suis*. In order to evaluate a possible involvement of the cytochrome *cbb3*-type and cytochrome *bd* oxidases in bacterial replication within the host cell, we examined the effect of *ccoN* and *cydB* deletions on the intracellular survival of *B. suis*. These genes, produced by PCR with primers CcoN5′ (GACGGCTACAGGATCAGC AAG) and CcoN3′ (CGGCGAATTCTTATTCGGCAGGCT GCATGG) and primers CydB5′ (CGGCAAGCTTGCAATG CGACGGACGAACAG) and CydB3′ (CGCTCTAGACCAA GCGCAGGCGCGATCAG) were cloned, and the 870-bp NaeI and 124-bp StyI fragments within the *ccoN* and *cydB* open reading frames, respectively, were deleted and replaced by the kanamycin or chloramphenicol resistance cassette. *B. suis* mutants were obtained as described for Δ*frnN*.

The multiplication rates of wild-type and mutant strains of *B. suis* 1330 were determined after infection of differentiated human macrophage-like THP-1 cells. Infection of 5×10^5 cells was performed in 24-well plates as indicated above, at a multiplicity of infection of 20. At 1.5, 7, 24, and 48 h p.i., cells were washed with PBS and lysed in 0.2% Triton X-100. CFU were determined by plating serial dilutions on TS agar.

Deletion of *cydB* resulted in a reduced multiplication rate of 0.27 ± 0.05 compared to that of the wild-type strain ($P = 0.01$; Student's *t* test) at 48 h p.i. (Fig. 3). Similar results were obtained with the murine macrophagic cell line J774.A1 (not shown), in agreement with results previously published for *B. abortus* (6). This rather small effect could be considered incompatible with our previous work showing that the *cydD* mutant was more severely affected than the *cydB* mutant (11). Deletion of *cydB*, the last gene of the *cydDCAB* operon, however, does not prevent expression of *cydD*. *cydDC* mutants of *E. coli* are pleiotropic and show highly reduced levels of all periplasmic cytochromes (19). A *cydD* deletion in *B. suis* may have a similar effect, since there is good sequence conservation of *cydDC* genes and their products (4).

The *ccoN* mutation had no effect on the replication of the bacteria inside the macrophages (Fig. 3). This is consistent with the absence of expression of the *ccoNOQP* promoter at the intracellular state. No significant reduction in intracellular survival was observed for the strain with *fnrN* deleted (not shown).

Conclusion. *Brucella* possesses a set of genes which allows the bacteria to adapt to low oxygen tension (16), among which are two operons encoding high-oxygen-affinity terminal oxidases. The cytochrome *cbb3*-type and cytochrome *bd* oxidases were found to have differential expression patterns in *B. suis*. The first one was specifically expressed in vitro, with maximal activation under microaerobiosis and dependent on FnrN. The second one was preferentially expressed during intracellular multiplication and was involved in adaptation to the replicative niche. Since the cytochrome *bd* oxidase was found to be repressed by FnrN, its constant expression rate within cells infected by the Δ*fnrN* strain indicated that FnrN was not involved in the regulation at the intracellular state. This was in accordance with the fact that this regulator was not necessary for multiplication of *B. suis* within cells. Intracellular expression of the cytochrome *bd* oxidase may indicate low oxygen tension

inside the macrophage. The affinities of *B. suis* cytochrome oxidases for oxygen have not yet been established, but in *Rhodobacter capsulatus*, which possesses both cytochrome *cbb3* and cytochrome *bd* oxidases, the latter was proposed to have the higher affinity (22). The important attenuation of the *cydB* mutant of *B. abortus* in mice (6) suggests that a very low oxygen tension is encountered by the pathogen in vivo. The use of the cytochrome *bd* oxidase most likely facilitates *Brucella* survival in the host, where the bacteria reside in various tissues previously described as containing low but variable oxygen concentrations (1, 15).

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